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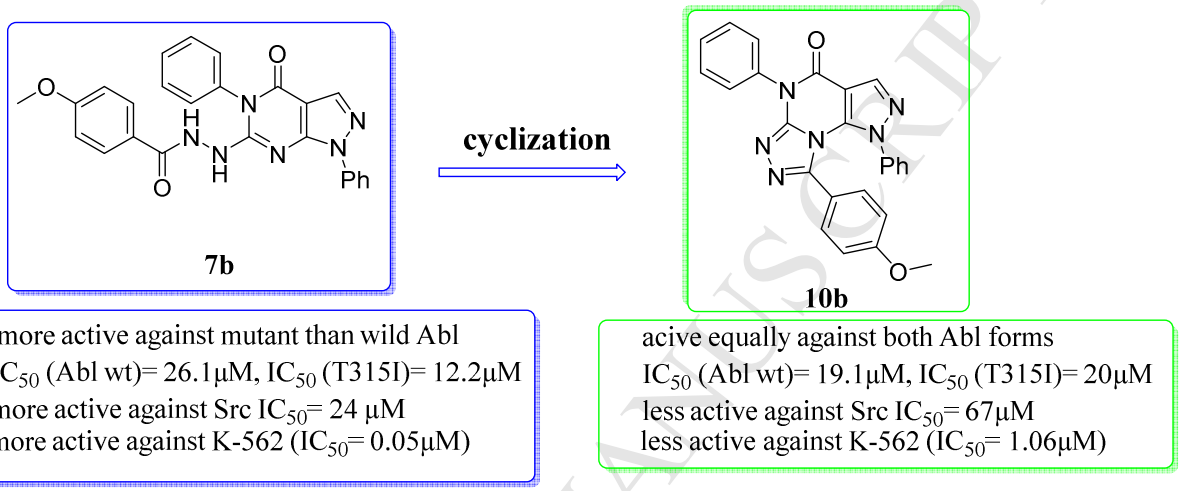

Novel pyrazolo[3,4-*d*]pyrimidines as dual Src-Abl inhibitors active against mutant form of Abl and the leukemia K-562 cell line

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Abstract

Some novel 6-substituted pyrazolo[3,4-*d*]pyrimidines **4**, **5**, **6a-d**, **7a-c**, **8** and pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*a*]pyrimidines **9a-c**, **10a-c**, **11**, **12a,b**, **13a-c** and **14** were synthesized and characterized by spectral and elemental analyses. They were screened for their biological activity *in vitro* against Abl and Src kinases. Compounds **7a** and **7b** revealed the highest activity against both wild and mutant Abl kinases as well as the Src kinase and the leukemia K-562 cell line. They can be considered as new hits for further structural optimization to obtain better activity.

Keywords: pyrazolo[3,4-*d*]pyrimidines, Src inhibition, Abl inhibition, K-562 cell line, molecular modeling.

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1. Introduction

Cancer is considered as a health burden worldwide due to its clinical and economic outcomes. It is the second leading cause of death after the cardiovascular diseases [1, 2]. The new cancer cases are expected to increase by as much as 15 million per year by 2020, according to the World Health Organization, unless further preventive measures are put into practice [3]. The Agency for Healthcare Research and Quality estimates that the direct medical costs (total of all health care expenditures) for cancer in the US in 2013 were $74.8 billion [1]. Chronic myeloid leukemia (CML) is a type of cancer that starts in certain blood-forming cells of the bone marrow. In CML, a genetic change takes place forming an abnormal gene called Bcr-Abl, which turns the cell into a CML cell. The leukemia cells grow and divide, building up in the bone marrow and spilling over into the blood. In time, the cells can also settle in other parts of the body, including the spleen. CML is a fairly slow growing leukemia, but it can also change into a fast-growing acute leukemia that is hard to treat [1]. The future of cancer treatment depends on development of targeted agents that specifically block key proteins involved in progression of specific types of cancer [4]. Therefore, the first-line treatment for CML was the selective inhibition of Bcr-Abl kinase activity by imatinib mesylate (Gleevec) **I**. However, resistance to Gleevec has been documented in patients through mutations both in and outside the Bcr-Abl kinase domain leading to interference with imatinib binding to Bcr-Abl [5]. As a consequence, there was a growing need for developing second-generation small molecule inhibitors able to treat Gleevec-resistant CML [6-9].

Second generation Abl inhibitors were synthesized through rational drug design approaches to overcome resistance to **I** [10]. Recently, Novartis disclosed Nilotinib (Fig.1, **II**), structurally related to **I**, which showed promising results in preclinical studies and was 10–25-fold more potent compared to **I** in cellular assays. Compound **II** also inhibited the growth of cell lines expressing Bcr-Abl mutants resistant to **I** [11]. On the other hand, unlike **I** and its derivatives, **III** (Fig. 1) interacted with the peptide substrate binding site of Abl and inhibited wild type kinase 10-fold more potently than **I**, and it also had activity against kinase domain mutations resistant to **I** [12].

Furthermore, many 4-amino-substituted pyrazolo[3,4-*d*]pyrimidines were synthesized and found to be active on different oncogenic tyrosine kinases and cancer cell lines

depending on the nature and position of substituents on the heterocyclic core [13−23]. Compound **IV** was an inhibitor of Abl (wild type) with K_i = 0.04 μM [22], while compound **V** had K_i = 0.025 μM on Abl T315I (mutant form) (Fig. 1) [14]. Although the most widely accepted mechanism for imatinib resistance is the presence of kinase domain mutations [5], cells from patients resistant to imatinib express an activated form of the (Src Family Kinases, SFKs) Lyn [24]. Moreover, Hck (another SFK) and Lyn are over-expressed and activated in CML blast-crisis patients and their up-regulation correlates with disease progression and resistance in cell lines and patients treated with imatinib [25, 26]. Therefore, compounds that can inhibit both Src and Abl might be useful in the treatment of patients who have relapsed on imatinib. Abl is known to share significant sequence homology with Src and in its active conformation it bears remarkable structural resemblance with most SFKs [27]. As a result, ATP-competitive compounds originally developed as Src inhibitors frequently exhibited potent inhibition of Abl kinase [28, 29]. Thus, several second generation Bcr-Abl kinase inhibitors targeting both Src and Abl kinases were synthesized in order to combat imatinib resistance, such as dasatinib **VI** and bosutinib **VII** (Fig.2) [30, 31]. Additionally, many pyrazolo[3,4-*d*]pyrimidines that were designed originally as Src inhibitors, exhibited potent inhibition of Abl kinase as PP2, **VIII** [32] and compound **IX** (Fig. 2) that revealed Ki values 0.22 and 0.19 μM against Src and Abl enzymes, respectively [19].

It was found that most of the reported compounds carried substitutions at positions 1, 4 and 6 of the pyrazolo[3,4-*d*]pyrimidine core, therefore, this work aimed to explore the effect of the substitution of a phenyl ring at position 5 in addition to the functional moieties at positions 1 and 6 hoping to develop a better structure activity relationship. Moreover, some pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*a*]pyrimidines were prepared to study the effect of this structure rigidification on the biological activity. Furthermore, compounds with promising activity on the enzyme have been screened against Leukemia K-562 cell lines. Moreover, the activity of the most active compound against both Abl and Src kinases has been validated through molecular modeling technique.

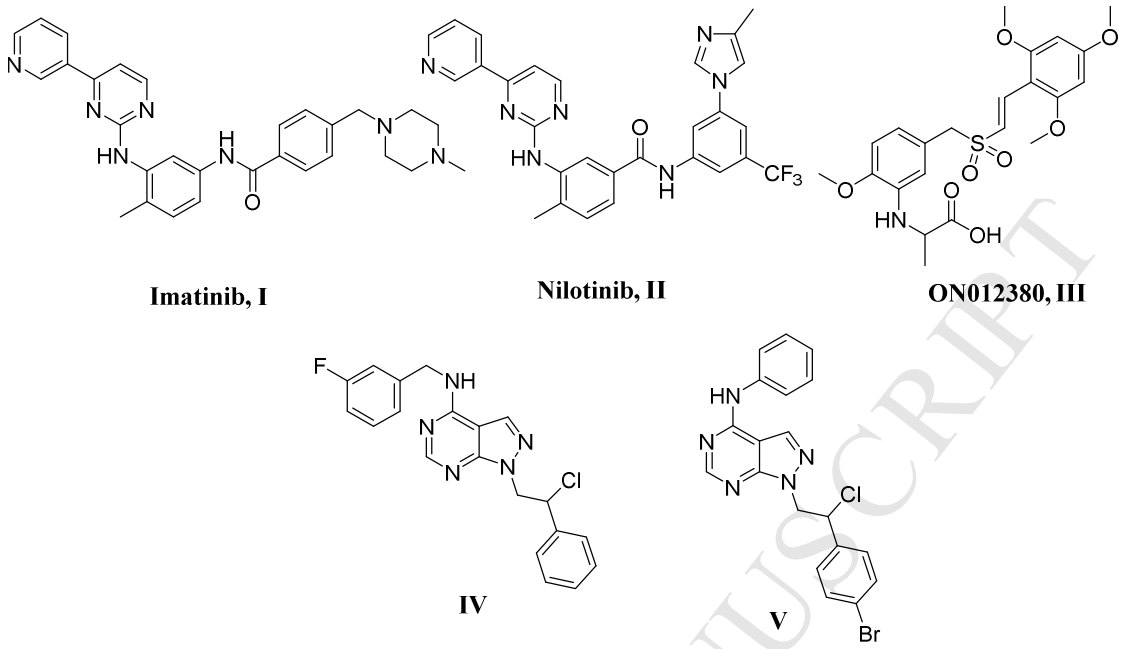


Fig. 1. Structures of some Abl inhibitors

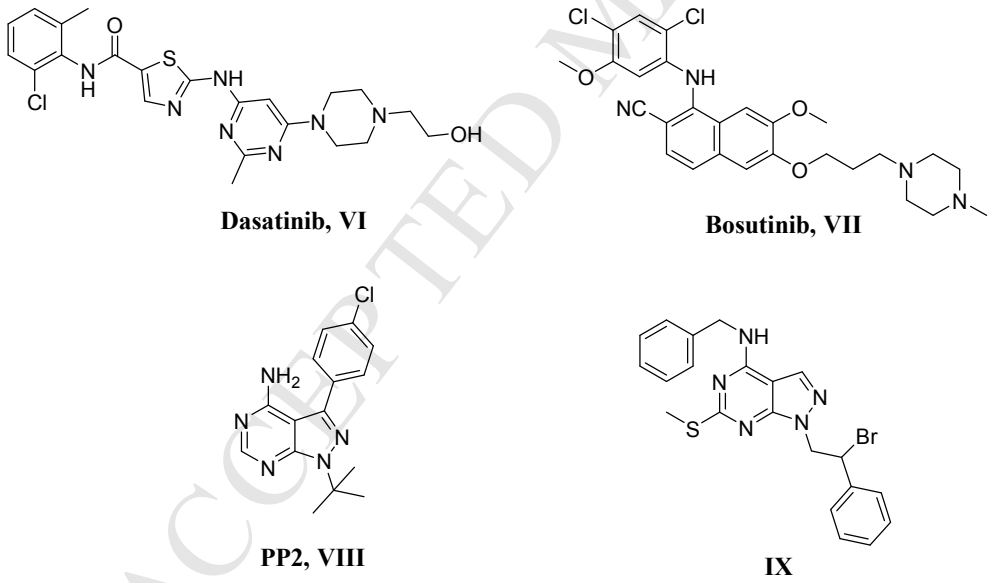


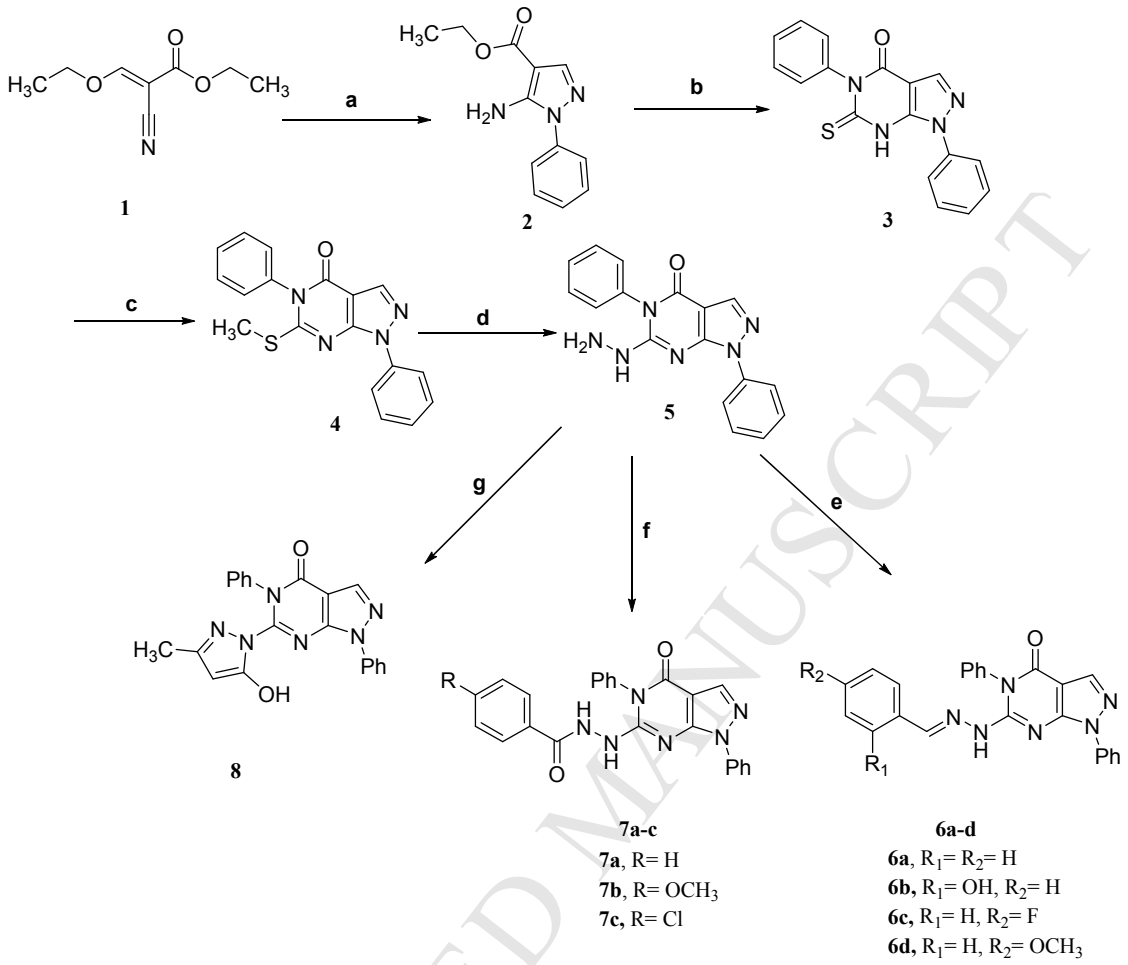
Fig. 2. Structures of some dual Abl-Src kinase inhibitors.

2. Results and discussion

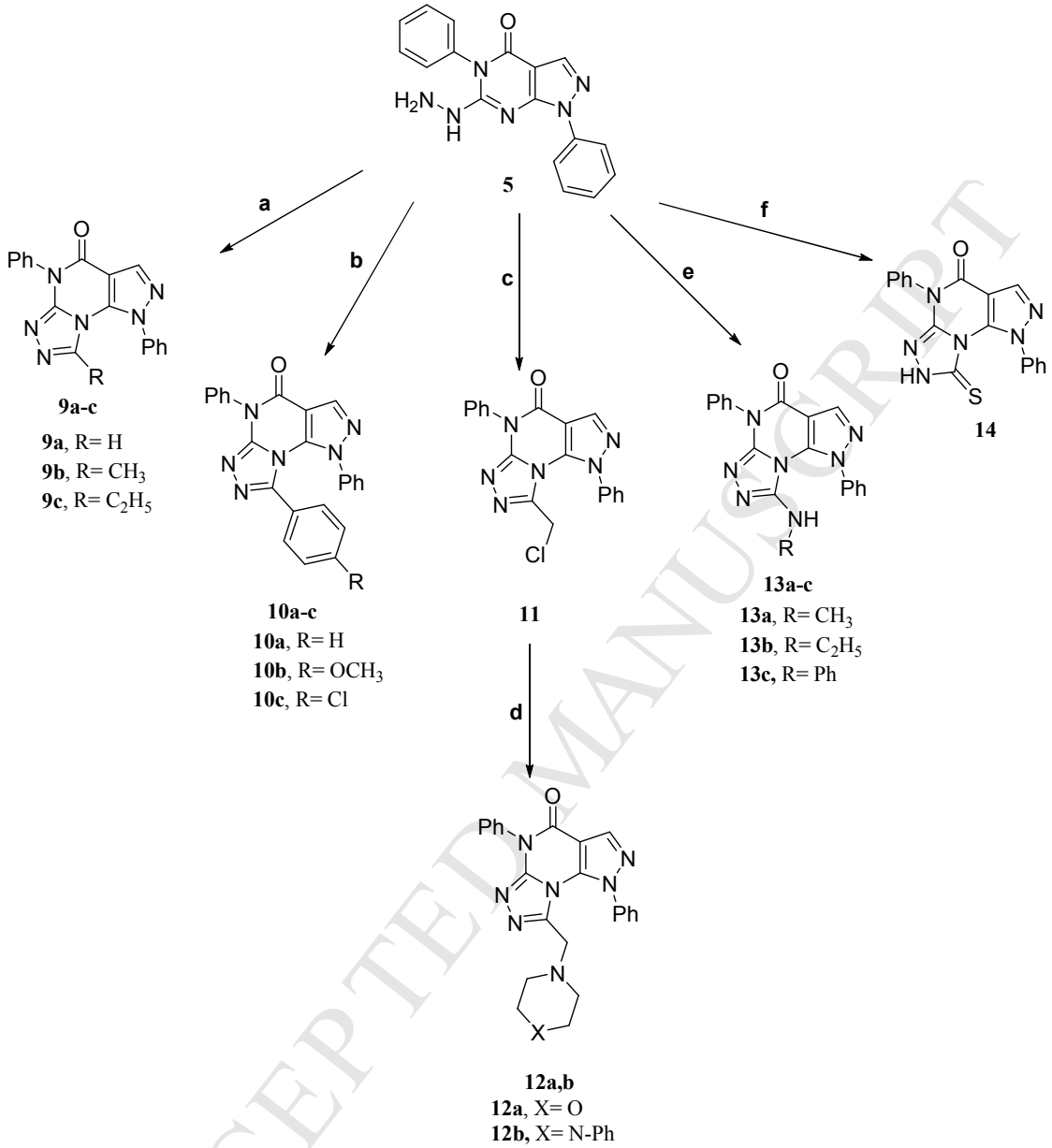
2.1. Chemistry

The target compounds were synthesized as outlined in schemes 1, 2. Ethyl ethoxy methylene cyanoacetate **1** [33] was reacted with phenyl hydrazine according to the reported procedure [34] to obtain ethyl 5-amino-1-phenyl-1*H*-pyrazolo-4-carboxylate **2.** The pyrazolo[3,4-d]pyrimidine core of **3** was formed from the reaction of **2** and phenyl isothiocyanate [35]. The reaction of **3** with methyl iodide in dry acetone in the presence of potassium carbonate afforded **4**. ^{1}H NMR spectrum showed the presence of a singlet signal at 3.75 ppm corresponding to the methyl group. Treatment of **4** with excess hydrazine hydrate in absolute ethanol resulted in 6-hydrazinyl derivative **5.** The IR spectrum of **5** demonstrated stretching bands at 3319, 3273 and 3207cm^{-1} corresponding to NH_2 and NH, respectively. Moreover, ^{1}H NMR of compound **5** showed two exchangeable signals at 5.64 and 9.68 corresponding to NH_2 and NH, respectively, along with the disappearance of the signal of the methyl group (Scheme 1). Condensation of **5** with different aldehydes in absolute ethanol afforded **6a-d.** ^{1}H NMR spectra of compounds **6a-d** revealed an increase in the integration of the aromatic protons indicating the presence of additional aromatic ring. The characteristic olefinic CH singlet signal appeared at range 7.47-9.08 ppm. Moreover, compound **6b** showed the presence of OH singlet signal at 10.08 ppm which disappeared upon deuteration. Compound **6d** revealed a singlet signal at 3.79 ppm corresponding to the methoxy group. Furthermore, the hydrazine derivative **5** was stirred with various benzoyl chlorides in the presence of pyridine at room temperature to obtain **7a-c**. ^{1}H NMR spectra of these compounds revealed an increase in the integration of the aromatic protons indicating the presence of an additional aromatic ring. Also, singlet signals appeared in the range of 9.74-13.17 ppm for the 2NH protons which disappeared upon deuterium exchange. In addition, ^{13}C NMR spectrum of **7a** and **7c** supported the carbon skeleton of the obtained structure and revealed the presence of an additional C=O signals at 171.3 and 169.2, respectively (Scheme1). Meanwhile, the target compound **8** was obtained via reflux a solution of **5** with ethyl acetoacetate in presence of anhydrous potassium carbonate in absolute ethanol. ^{1}H NMR of compound **8** showed the presence of a singlet signal at 2.41 ppm due to the methyl group, ^{13}C NMR spectrum showed signals at 12.3 and 152.4 corresponding to

CH_3 of the pyrazole and C=O of the pyrimidinone, respectively. Additionally, the mass spectrum exhibited the molecular ion peak at m/z 384.18. The pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*a*]pyrimidines were prepared according to scheme 2 where the hydrazine derivative 5 was treated with different aliphatic carboxylic acids or benzoyl chloride derivatives to afford **9a-c** and **10a-c**, respectively. ^{1}H NMR spectra of **9a-c** showed the absence of NH and NH_2 signals**.** Compound **9b** displayed a singlet signal at 2.40 ppm corresponding to the methyl group while **9c** revealed triplet-quartet signals at 1.33 ppm and 2.78 ppm respectively, due to the ethyl group. On the other hand, ^{1}H NMR spectra of **10a-c** showed an increase in the integration of the aromatic protons indicating the presence of an additional aromatic moiety. Moreover, a singlet peak at 3.79 ppm revealed the presence of methoxy group for compound **10b**. The ^{13}C NMR was in good agreement with the molecular formula of compounds **10a** and**10b**, that, the presence of methoxy signal at 55.9 and C=O signal at 162.0 confirmed the carbon backbone of **10b**. Similarly, the reaction of 5 with chloroacetyl chloride in glacial acetic acid resulted in 8-chloromethyl derivative **11.** ^{1}H NMR showed a singlet peak downfielded to 4.66 ppm corresponding to methylene group. Compounds **12a**,**b** were obtained by treatment of **11** with morpholine and 4-phenyl piperazine, respectively in dry dioxane in the presence of anhydrous potassium carbonate. ^{1}H NMR showed the presence of the methylene spacer which upfielded to the range of 3.65-3.71 ppm in comparison to 4.65 ppm of the starting **11**. Also, multiple aliphatic signals with integration of eight protons confirmed the introduction of the new alicyclic amine. Additional phenyl ring was observed by increased integration of the aromatic region for the compound **12b.** It is noteworthy that the reaction of **5** with different isothiocyanates in pyridine afforded **13a-c** without isolation the thiosemicarbazide intermediates. The suggested mechanism of this reaction could be illustrated in Figure 3. Additionally, the formation of cyclic compounds could be confirmed by evolution of H_2S during the reaction which stained the lead acetate paper with black color.



Scheme 1. Reagents and reaction conditions: (a) $PhNHNH_2$, absolute ethanol, reflux, 15 h.; (b) phenyl isothiocynate, pyridine, reflux, 12h.; (c) CH_3I, K_2CO_3, acetone, reflux, 8h.; (d) 80% NH_2NH_2, absolute ethanol, reflux 12h.; (e) app.aldehyde, absolute ethanol, reflux.; (f) app. benzoyl chloride, pyridine, RT.; (g) ethylacetoacetate, K_2CO_3, ethanol, reflux 11h.



Scheme 2. Reagents and reaction conditions: (a) excess of aliphatic acid, reflux; (b) app. benzoyl chloride, dry pyridine, reflux; (c) chloroacetyl chloride, acetic acid, reflux 4h.; (d) morpholine or phenylpiperazine, KOH, dry dioxane, reflux; (e) app. isothiocyanate, pyridine, reflux; (f) C_2S, KOH, absolute ethanol, reflux 6h.

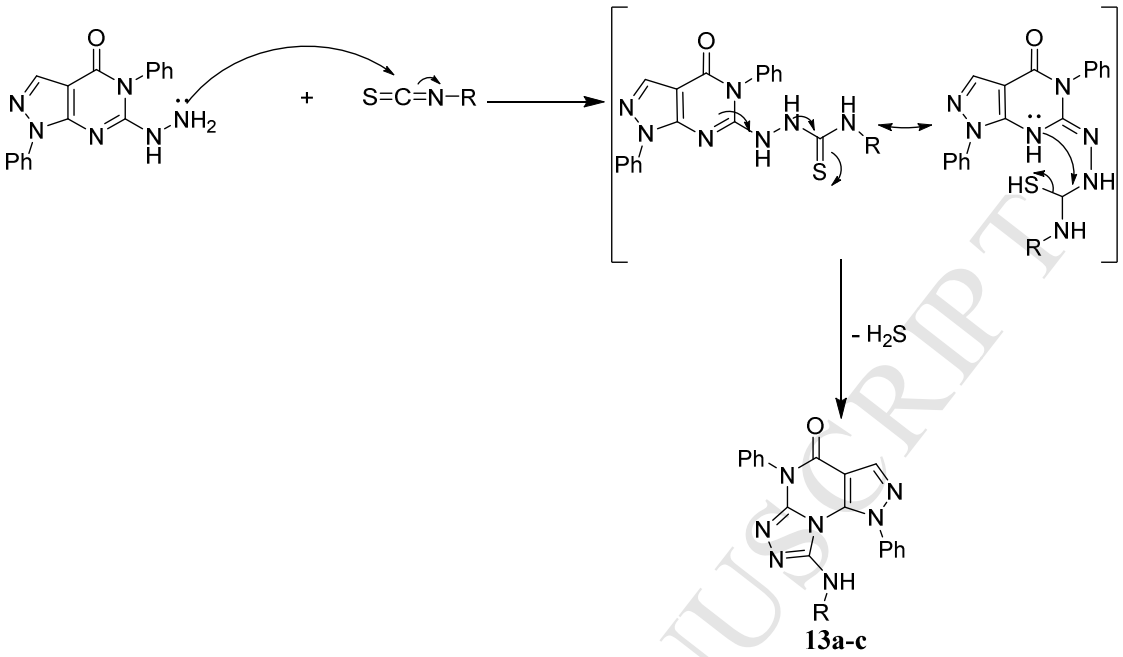


Fig.3. The proposed mechanism for the formation of **13a-c.**

^{1}H NMR spectra revealed the presence of one exchangeable signal of NH at 6.26, 6.85 and 9.10 ppm for **13a**, **13b** and **13c**, respectively. ^{1}H NMR spectrum for **13a** showed a singlet peak at 2.88 ppm corresponding to the methyl group**.** Triplet-quartet pattern exploited the presence of ethyl group in **13b** while an additional aromatic ring in **13c** could be deduced from the increase in Ar-H integration**.** Finally, the reaction of **5** with carbon disulfide in absolute ethanol in the presence of potassium hydroxide resulted in **14** (Scheme 2). ^{1}H NMR spectrum was in good agreement with the IR spectrum, in addition, the presence of a signal at 167.8 ppm corresponding to C=S confirmed the obtained structure. Mass spectrum displayed the molecular ion peak consistent with its molecular weight.

2.2. *In vitro* enzyme inhibitory assay

All the target compounds were evaluated for their enzyme inhibitory activity in *in-vitro* assays against wild type Abl (wt). First, Abl (wt) was treated with the compounds at a concentration of 100 µM and the % residual Abl (wt) activity of the selected compounds was summarized in Table 1. Second, the most promising compounds at 100 µM (**7a**, **7b**, **10a** and **10b**) have been further tested against the kinases Src and PI3K and their % residual activity was presented in Table 2. Moreover, the most active compounds **7a, 7b** and **10b** were also screened against the mutant Abl tyrosine kinase (T315I), and their IC_{50} are represented in Table 3.

Table 1.% Residual Abl (wt) activity of compounds at 100 µM.

Compd. ID	% activity Abl (wt) at 100 µM	Compd. ID	% activity Abl (wt) at 100 µM
4	84.0 ± 9.0[a]	**9b**	71.0 ± 8.0
5	46.0 ± 6.0	**9c**	70.0 ± 7.0
6a	47.0 ± 7.0	**10a**	28.0 ± 3.0
6b	96.0 ± 10.0	**10b**	17.0 ± 2.0
6c	36.0 ± 4.0	**10c**	68.0 ± 7.0
6d	77.0 ± 8.0	**11**	68.0 ± 7.0
7a	6.3 ± 0.5	**13a**	>100.0
7b	23.0 ± 2.0	**13b**	>100.0
7c	26.0 ± 3.0	**13c**	33.0 ± 5.0
8	98.0 ± 8.0	**14**	66.0 ± 8.0
9a	39.0 ± 4.0	**Dasatinib**	< 1.0

[a]values are the mean of two independent replicates ±S.D.

Table 2. % Residual Src and PI3K activity of selected compounds at 100 µM

Compd. ID	% activity Src at 100 µM	% activity PI3K at 100 µM
7a	7 ± 1[a]	100 ± 11
7b	20 ± 4	80 ± 10
10a	50 ± 4	82 ± 6
10b	35 ± 3	90 ± 3

[a]values are the mean of two independent replicates ±S.D.

Table 3: The IC_{50} (µM) of selected compounds toward Src, Abl (wt) and T315I Abl and their (cytotoxic concentration) CC_{50} against K-562 cell line.

Compd. ID	**IC_{50}(µM)** ±SD[b] **(Src)**	**IC_{50}(µM)** ±SD[b] **(Abl wt)**	**IC_{50}(µM)** ±SD[b] **(Abl T315I)**	**CC_{50} (µM)** ±SD[c] Leukemia K-562 Cell line
5	n.d[a]	n.d[a]	n.d[a]	2.3700 ± 0.0800
6c	n.d[a]	110.0000 ± 10.0000	n.d[a]	4.0600 ± 0.1400
7a	4.7000 ± 0.7000	20.9000 ± 0.9000	13.8000 ± 0.8000	0.0700 ± 0.0020
7b	24.0000 ± 2.0000	26.1000 ± 0.5000	12.2000 ± 0.5000	0.0500 ± 0.0010
7c	n.d[a]	65.6000 ± 0.6000	n.d[a]	0.8600 ± 0.0200
10a	n.d[a]	40.0000 ± 5.0000	n.d[a]	0.9200 ± 0.0300
10b	67.0000 ± 8.0000	19.0000 ± 1.0000	20.0000 ± 2.0000	1.0600 ± 0.0500
13c	n.d[a]	150.0000 ± 10.0000	n.d[a]	4.9200 ± 0.1600
PP2[d]	0.1800 ± 0.0500	0.5000 ± 0.2000	n.d[a]	24.5000 ± 0.7500
Dasatinib	n.d[a]	0.0040 ± 0.0002	0.0800 ± 0.0200	0.2500 ± 0.0200

[a] n.d = not determined
[b] values are the mean of two independent replicates ±S.D.
[c] values are the mean of tetrareplicates ±S.D
[d] PP2, pyrazolopyrimidine Src/Abl inhibitor [19]

From the obtained results (Tables 1, 2 and 3) it was observed that among the tested compounds, only compounds**7a**, **7b** and **10b** are the most active against the wild type Abl. Compounds **7a** and **7b** also displayed comparable activity against Src, but not against PI3K. Interestingly, *p*-methoxybenzohydrazide **7b** and its unsubstituted analog **7a** revealed also better activity against mutant Abl (T315I) with IC_{50} equal to 12.2 and 13.8 µM, respectively. Compound **10b**, the cyclized form of **7b**, also maintained the same activity against the mutant as observed against the wild type (IC_{50} of 20 µM and 19.1 µM, respectively). On the other hand, the cyclization of **7b** into **10b** resulted in decreased the activity against Src kinase with IC_{50} values of 24 and 67 µM, respectively. Thus, compounds **7a**, **7b** and **10b** seem to be resilient towards the resistance induced by the gatekeeper mutation T315I. Their chemical scaffolds could be further exploited to develop dual Src-Abl inhibitors, also active against the clinically relevant Abl mutant T315I.

2.3. In vitro cytotoxic activity against Leukemia K-562 cell line

Eight compounds that showed promising IC_{50} values against Abl enzyme (wt) were tested for their cytotoxic activity against the leukemia cell line K-562. This screening was performed at Vacsera, Cairo, Egypt and the results are summarized in Table 3. The tested compounds revealed remarkable activity with cytotoxic concentration CC_{50} ranging from 0.05 to 4.92 μM. Moreover, from the obtained results, structure activity relationships revealed that acylation of compound **5** with unsubstituted or 4-methoxybenzoyl chloride resulted in the most active derivatives **7a** and **7b**, respectively. On the other hand, the 4-chloro derivative **7c** revealed a lower activity. Thus, the presence of an electron donating group is more favorable than an electron withdrawing moiety. On the other hand, conversion of the hydrazine derivative **5** into 4-fluorobenzohydrazone **6c** resulted in decrease in the activity. Furthermore, 8-aryl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*a*]pyrimidine-4(5*H*)-ones **10a,b** exhibited higher activity than the phenylamino congener **13c.** Moreover, cyclization of **7a,b** into **10a,b** resulted in decrease in the cytotoxic activity. In general, the CC_{50} values exerted towards the K-562 cell line were lower than the corresponding IC_{50} values towards the single recombinant Src or Abl enzymes (Table 3). However, the effects of the compounds at the cellular level may be amplified through signal transduction cascades activated in response to the simultaneous inhibition of both Src and Abl activity, resulting in a synergic effect. In addition, differences between *in vitro* enzymatic assays and cellular viability measurements may also account for these discrepancies. For example, the absolute enzyme concentrations are surely different between *in vitro* and cellular tests.

2.4. Molecular modeling

Molecular docking technique was used to interpret the observed promising activity of compound **7b** on both Abl types. Therefore, docking of **7b** into the three dimensional X-ray crystal structure of the wild type human Bcr-Abl kinase (PDB code: 4WA9) and at the crystal structure of human Abl T315I gatekeeper mutant form (PDB code: 4TWP) [36] both in complex with axitinib were carried out using Accelry's Discovery Studio software package [37]. The selection of these X-ray resolved complexes for the docking study was based on providing a comparative perspective for

the conformational changes taking place in the enzyme upon ligand binding. Recently, axitinib was shown to bind the wild form of Abl kinase in the inactive state known as "DFG out" in which the catalytic Asp381 is oriented away from the ATP binding site while Phe382 is oriented towards the binding site [38]. Regarding the mutant form, axitinib was shown to bind to the active state known as "DFG in" where the Asp381 is oriented towards the ATP binding site while Phe382 is oriented inwards. The validity of the docking protocol was ensured by re-docking of the co-crystallized axitinib into the active site of both forms. The coordinates of the best scoring docking pose of axitinib was compared with its coordinates in the co-crystallized PDB files based on the binding mode and root mean square deviation (rmsd). The re-docked ligand showed an rmsd of 0.938 Aº with CDOCKER interaction energy of -55.3216 at the wild type Bcr-Abl kinase and an rmsd of 0.713 Aº with CDOCKER interaction energy of -58.2649 at the mutant form (Figure 4, 5). In both, axitinib was forming two hydrogen bonds with the amino acids of the hinge region Glu316 and Met318. Two π-π interactions were formed with Phe317 and additional hydrogen bonds with Tyr253 and Lys271 were also reported. In the wild form of Abl kinase, the methoxy substituted phenyl in **7b** was extending towards the hinge region where its methoxy group was engaged in a hydrogen bond with the crucial Met318 (Figure 6). Multiple pi interactions between Phe382 and both pyrazolopyrimidine and its 1-phenyl moiety was reported while the 5-phenyl moiety was forming a cation-pi interaction with Lys271.

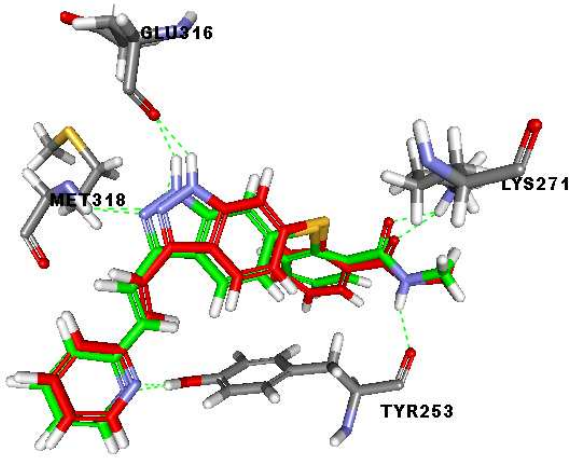


Fig. 4. Superimposition of the co-crystallized 3D structure of axitinib (green) and its top docking pose (red) in the active site of the wild type Bcr-Abl kinase (PDB: 4WA9)

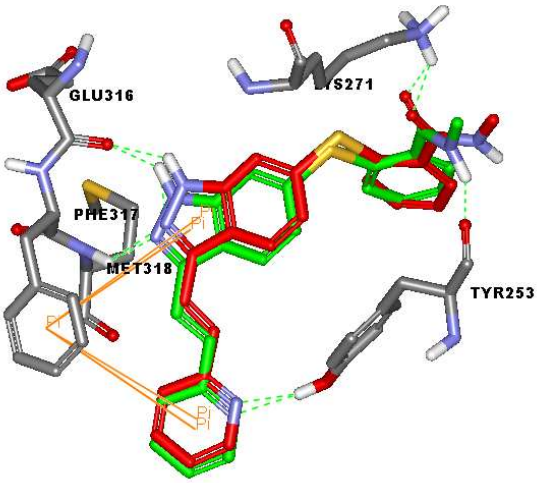


Fig. 5. Superimposition of the co-crystallized 3D structure of axitinib (green) and its top docking pose (red) in the active site of mutant Bcr-Abl kinase (PDB: 4TWP)

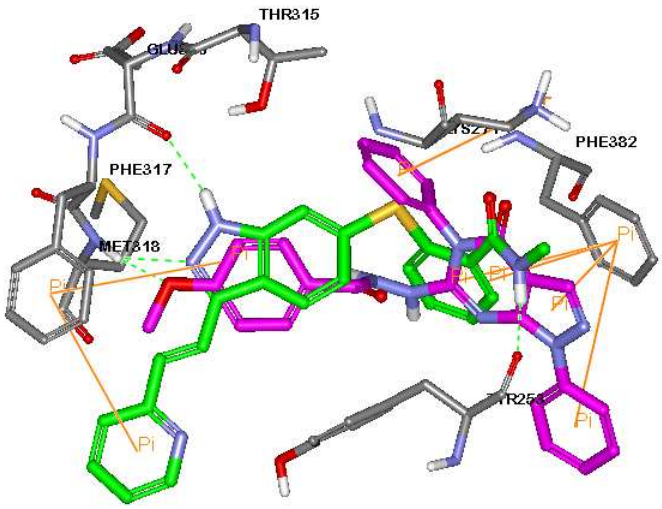


Fig. 6. Overlay of axitinib (green) and compound **7b (magenta)** in the active site of the wild type Bcr-Abl kinase (PDB: 4WA9)

An interesting feature in the top docking pose of **7b** at the active site of T315I mutant, as compared to the wild form, was the flipping of the molecule in a fashion that allowed a good alignment with that of axitinib binding fashion (Figure 7). The pyrazole ring was engaged in one hydrogen bond with Met318. The pyrazole and the 1-phenyl ring were forming pi interactions with Phe317. Since Phe382 in the T315I mutant form was oriented away from the ATP binding site, its involvement in pi interactions with **7b** as in the wild enzyme is not expected. This change in the relative position of Phe382 may also result in providing a larger room to accommodate the molecule in a binding mode similar to that of axitinib. It is worth mentioning that neither Thr315 in the wild nor Ile315 in the mutant forms were involved in any interactions with compound **7b**.

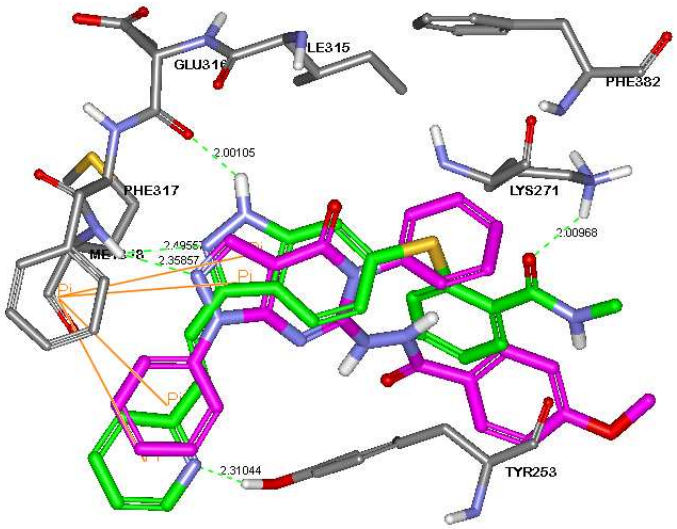


Fig. 7. Overlay of the best docking pose of compound **7b** (magenta) and axitinib (green) in the active site of T315I mutant Bcr-Abl kinase (PDB:4TWP)

The three dimensional X-ray crystal structure of the kinase domain of c-Src in complex with a substituted pyrazolopyrimidine (PDB code: 4O2P) was used for molecular docking of compounds **7a** and **7b** [39]**.** The selection of this x-ray resolved complex was based on the structural similarity between the compounds **7a** and **7b** with the co-crystalized ligand. The co-crystallized ligand interacted with the kinase domain of c-src through pi interactions with Tyr 340 and Lys 295 and hydrogen bonds with Met 341 and the gate keeper Thr 338 (Fig.8).

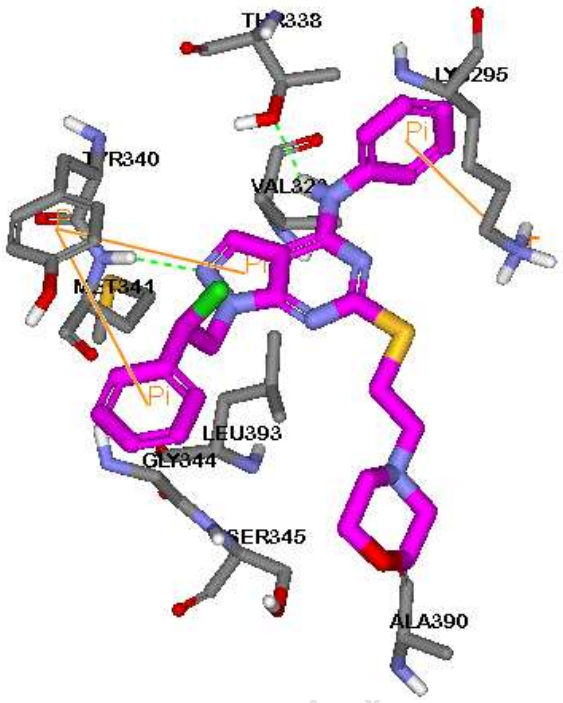


Fig. 8. The cocrystallized pyrazolopyrimidine in c-Src (PDB code: 4O2P)

The best docking poses of compounds **7a** and **7b** in the kinase domain of c-Src revealed a hydrogen bond between Lys 295 and the carbonyl moiety in the core of compounds **7a** and **7b**. Lys 295 was also involved in a cation-pi interaction with the phenyl ring. Tyr 340 in the hinge region interacted with the phenyl ring in **7a** or the methoxy substituted phenyl in **7b** through a pi-pi interaction. The electron donating nature of the methoxy group in **7b** is hypothized to decrease the aromatic interaction with the electron rich phenyl ring of Tyr 340. Met 341 was in close proximity to the hydrazide oxygen in both compounds. The target compounds **7a** and **7b** were not involved in interaction with the THR338 (Fig. 9, 10). It is worthy to mention that the pyrazolopyrimidine core in **7a** and **7b** was flipped relative to the native co-crystallized ligand.

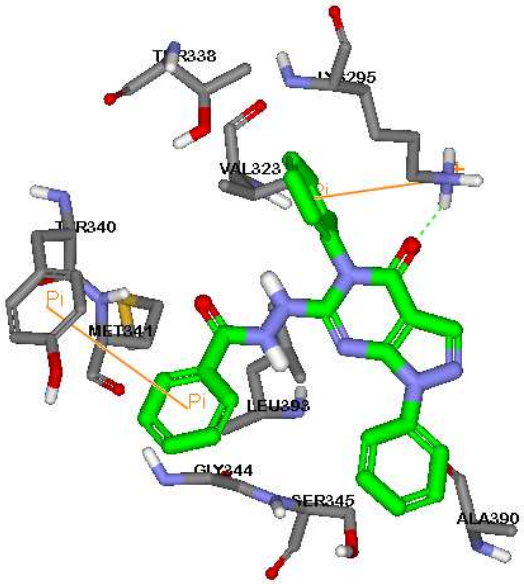


Fig. 9. The best docking pose of compound **7a** in the active site of the kinase domain of c-Src(PDB: 4O2P)

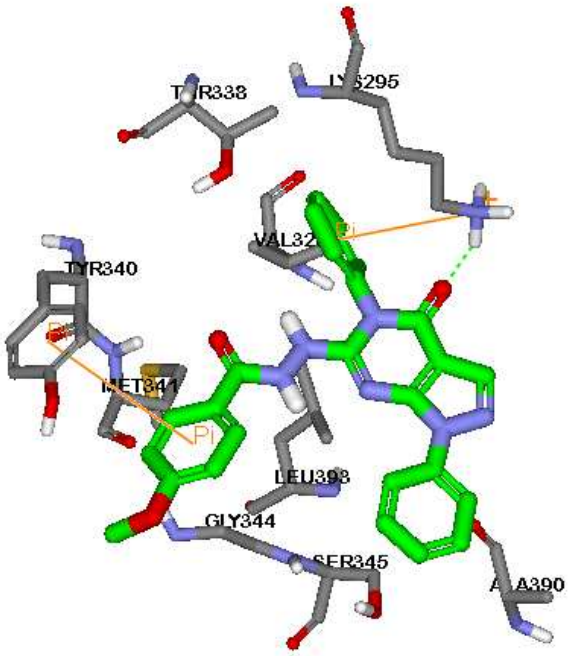


Fig. 10. The best docking pose of compound **7b** in the active site of the kinase domain of c-Src (PDB: 4O2P)

3. Conclusion

Some novel pyrazolo[3,4-*d*]pyrimidines and pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*a*] pyrimidines were synthesized through facile procedures. They revealed mild to moderate activity against both Src and Abl (wt) kinases. Compounds **7a**, **7b** and **10b** were the most active compared to the other derivatives against both enzymes and the mutant Abl type T135I, and the leukemia K-562 cell line. However, one point of interest in the present study, is the identification of chemical scaffolds (compounds **7a**, **7b** and **10b**), which were unaffected by the T315I mutation and can be further exploited in order to develop specific inhibitors for this clinically relevant mutant.

4. Experimental

4.1. Chemistry

Melting points were determined by open capillary tube method using Electrothermal 9100 melting point apparatus and are uncorrected. Elemental microanalyses were performed at the Regional Center for Mycology and Biotechnology, Al-Azhar University. The infrared spectra (IR) were recorded as potassium bromide discs on Schimadzu FT-IR 8400S and Bruker FT-IR 12243136 spectrophotometers at Faculty of Pharmacy, Cairo University and Central Research Lab, Nahda University, respectively. ^{1}H NMR spectra were recorded on Bruker spectrophotometer at 400 MHz. Chemical shift values (δ) are given in parts per million (ppm) downfield from tetramethylsilane (TMS) as internal reference. ^{13}C NMR spectra were recorded using Bruker, 100 MHz NMR spectrometer at Microanalytical Unit, Faculty of Pharmacy, Cairo University.Mass spectra were performed on Fennigan MAT, SSQ 7000 mass spectrophotometer at 70eV.Reactions were followed up by thin layer chromatography (TLC), using Silica gel/TLC cards DC-Alufolien-Kiesel gel with fluorescent indicator UV254 using chloroform or hexane : ethyl acetate 6 : 4 as the eluting system and the spots were visualized using VilberLourmet ultraviolet lamp at ג=254nm.

Compounds **1** [33], **2** [34], **3** [35] were prepared according to reported procedures.

4.1.1.6-(Methylthio)–1,5-diphenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one ***4***

A mixture of **3** (3.20 g, 10 mmol) and anhydrous potassium carbonate (1.65 g, 12 mmol) in dry acetone (50 mL) was stirred at room temperature for 1 h., then methyl iodide (7.10 g, 50 mmol) was added dropwise and the mixture was heated under reflux for 8 h. The solvent and the excess methyl iodide were evaporated under reduced pressure and the remaining residue was poured onto ice cold water. The separated product was crystallized from acetone to obtain white crystalline solid. Yield %: 76 (2.43 g), m.p.: 232-234°C. IR $\upsilon_{max,}$ cm^{-1}: 3053 (Ar-CH), 2954 (aliph-CH), 1701 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 3.75 (s, 3H, -SCH_3), 7.14 (d, 1H, *J* = 7.56 Hz, Ar-H), 7.29 (d, 1H, *J* = 7.88 Hz, Ar-H), 7.41- 7.54 (m, 3H, Ar-H), 7.81 (t, 1H, *J* = 7.40 Hz, Ar-H), 8.01 (t, 2H, *J* = 7.92 Hz, Ar-H), 8.64 (d, 2H, *J* = 8.00, Ar-H), 8.69 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO- d_6, 100 MHz): 43.0 (S-CH_3), 103.7 ($C4_{pyrazole}$), 121.3, 125.3, 125.7, 127.0, 127.7, 128.3, 129.5, 129.6, 129.7, 137.1, 137.2, 139.1, 146.3, 151.8, 157.0 (Ar-C), 158.4 (C=O). Anal.Calcd. for $C_{18}H_{14}N_4OS$ (334.09): C, 64.65; H, 4.22; N, 16.75. Found: C, 64.81; H, 4.29; N, 17.01.

4.1.2. *6-Hydrazinyl-1,5-diphenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one* ***5***

To a solution of compound **4** (3.34 g, 10 mmol) in ethanol (50 mL), hydrazine hydrate (80%) (0.1 g, 20 mmol) was added and the reaction mixture was refluxed for 12 h. The reaction mixture was cooled and the formed precipitate was filtered, washed with ethanol to obtain V as a white powder. Yield 71% (2.25 g), m.p.: 201-203°C. IR $\upsilon_{max,}$ cm^{-1}: 3319, 3273 (NH_2), 3207 (NH), 3082 (Ar-H), 1699 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 5.64 (s, 2H, NH_2, exchanged with D_2O), 7.14 (t, 1H, *J* = 7.34 Hz, Ar-H), 7.34 (t, 1H, *J* = 7.38 Hz, Ar-H), 7.40 (t, 2H, *J* = 7.86 Hz, Ar-H), 7.52 (t, 2H, *J* = 7.90 Hz, Ar-H), 7.79 (d, 2H, *J* = 7.76 Hz, Ar-H), 8.09 (d, 2H, *J* = 7.76 Hz, Ar-H), 8.18 (s, 1H, $H3_{pyrazole}$), 9.68 (s, 1H, NH, exchanged with D_2O). MS m/z (%): 318.13 (M^+, 2.66), 77.05 (100.00). Anal.Calcd. for $C_{17}H_{14}N_6O$ **(**318.12): C, 64.14; H, 4.43; N, 26.40. Found: C, 64.31; H, 4.49; N, 26.71.

4.1.3. General procedure for preparation of ***6a-d***

A mixture of equimolar amounts of compound **5** and the appropriate aromatic aldehyde (10 mmol) in absolute ethanol (50 mL) was refluxed for 4-7 h. The separated solid was filtered while hot and washed with ethanol to give the corresponding hydrazone product.

4.1.3.1. 6-[2-(Benzylidene)hydrazinyl]-1,5-diphenyl-1,5-dihydro-4H-pyrazolo[3,4-d] pyrimidin-4-one ***6a***

The reaction time was 7 h, yield 65% (2.64 g), m.p.: 160-164°C. IR $\upsilon_{max,}$ cm^{-1}: 3421 (NH), 3059 (Ar-H), 1724 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.30 (t, 1H, *J* = 7.36 Hz, Ar-H), 7.44-7.59 (m, 14H, 12Ar-H, NH exchanged with D_2O, and –CH=N), 8.02 (d, 2H, *J* = 7.92 Hz, Ar-H), 8.46 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO, 100 MHz): 103.2 ($C4_{pyrazole}$), 121.1, 125.3, 126.9, 128.5, 129.3, 129.5, 130.1, 130.4, 131.9, 132.9, 137.1, 139.1, 151.4 (Ar-C), 151.6 (C=O), 152.4 (C=N). MS m/z (%): 406.21 (M^+, 4.13), 404.21 (100.00). Anal.Calcd. for $C_{24}H_{18}N_6O$ **(**406.44): C, 70.92; H, 4.46; N, 20.68. Found: C, 71.08; H, 4.52; N, 20.89.

4.1.3.2. 6-[2-(2-Hydroxybenzylidene)hydrazinyl]-1,5-diphenyl-1,5-dihydro-4H-pyrazolo [3,4-d]pyrimidin-4-one ***6b***

The reaction time was 5h, yield 72% (3.04 g), m.p.: 186-187°C. IR $\upsilon_{max,}$ cm^{-1}: 3213 (br-OH), 3196 (NH), 3062 (Ar-CH), 1687 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 6.73 (t, 1H, *J* = 7.48 Hz, Ar-H), 6.85 (d, 2H, *J* = 8.68 Hz, Ar-H), 7.02 (m, 1H, Ar-H), 7.13-7.24 (m, 3H, Ar-H), 7.32-7.50 (m, 2H, Ar-H), 7.56 (t, 1H, *J* = 7.86 Hz, Ar-H), 7.65-7.69 (m, 2H, Ar-H), 8.07-8.09 (m, 3H, N=CH + 2 Ar-H), 8.21 (s, 1H, $H3_{pyrazol}$), 9.18 (s,1H, NH, exchanged with D_2O), 10.08 (s, 1H, OH, exchanged with D_2O). ^{13}C NMR (DMSO- d_6, 100 MHz): 103.5 ($C4_{pyrazole}$), 116.3, 119.8, 121.2, 122.3, 123.8, 125.5, 127.4, 128.9, 129.5, 131.2, 136.4, 136.7, 151.9, 153.7 (Ar-C), 152.4 (C=O), 155.8 (C=N). Anal.Calcd. for $C_{24}H_{18}N_6O_2$ **(**422.44): C, 68.24; H, 4.29; N, 19.89. Found: C, 68.43; H, 4.35; N, 20.04.

4.1.3.3. 6-[2-(4-Fluorobenzylidene)hydrazinyl]-1,5-diphenyl-1,5-dihydro-4H-pyrazolo [3,4-d]pyrimidin-4-one ***6c***

The reaction time was 7h, yield 61% (2.59 g), m.p.: 222-223°C. IR $\upsilon_{max,}$ cm^{-1}: 3332 (NH), 3059 (Ar-CH), 1712 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 6.81 (d, 1H, *J* = 9.44 Hz, Ar-H), 7.14-7.28 (m, 2H, Ar-H), 7.30-7.49 (m, 4H, Ar-H), 7.53-7.57 (m, 3H, CH=N + Ar-H), 7.64-7.69 (m, 2H, Ar-H), 8.07 (d, 2H, *J* = 7.88 Hz, Ar-H), 8.18 (dd, 1H, *J* = 5.95, 6.08 Hz, Ar-H), 8.21 (s, 1H, $H3_{pyrazole}$), 9.20 (s, 1H, NH exchanged with D_2O). ^{13}C NMR (DMSO- d_6, 100 MHz): 104.2 ($C4_{pyrazole}$), 116.1, 116.3, 121.0, 121.5, 121.6, 124.5, 127.1, 128.8, 129.5, 129.6, 130.1, 130.2, 138.9, 148.0, 150.0 (Ar-C), 152.7 (C=O), 154.8 (C=N). MS m/z (%): 424.18 (M^+, 11.63), 186.08 (100.00). Anal.Calcd. for $C_{24}H_{17}FN_6O$ **(**424.43): C, 67.92; H, 4.04; N, 19.80. Found: C, 68.13; H, 4.12; N, 19.97.

4.1.3.4. 6-[2-(4-Methoxybenzylidene)hydrazinyl]-1,5-diphenyl-1,5-dihydro-4H-pyrazolo [3,4-d]pyrimidin-4-one ***6d***

The reaction time was 4h, yield 81% (3.53 g), m.p.: 214-215°C. IR $\upsilon_{max,}$ cm^{-1}: 3321 (NH), 3035 (Ar-CH), 2953 (aliph-CH), 1660 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 3.79 (s, 3H, OCH_3), 7.01 (d, 2H, *J* = 8.88 Hz, Ar-H), 7.29 (t, 1H, *J* = 7.38 Hz, Ar-H), 7.43-7.47 (m, 5H, 4 Ar-H, and NH), 7.58 (s, 1H, N=CH), 7.60 (br s, 5H, Ar-H), 8.01 (d, 2H, *J* = 7.88 Hz, Ar-H), 8.43 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO- d_6, 100 MHz): 55.9 (OCH_3), 102.0 ($C4_{pyrazole}$), 114.9, 121.1, 122.3, 124.3, 126.7, 127.0, 128.9, 129.5, 130.4, 136.6, 138.3, 139.1, 151.1, 151.7, 162.1 (Ar C), 157.4 (C=O), 161.0 (C=N). MS m/z (%): 436.39 (M^+, 1.18), 44.03 (100.00). Anal.Calcd. for $C_{25}H_{20}N_6O_2$ **(**436.47): C, 68.80; H, 4.62; N, 19.25. Found: C, 68.97; H, 4.73; N, 19.39.

4.1.4. General procedure for preparation of ***7a-c****.*

A solution of compound **5** (3.18 g, 10 mmol) and the appropriate benzoyl chloride (10 mmol) in pyridine (20 mL) was stirred at room temperature for 3-6 h. The reaction mixture was poured onto cold water and neutralized with acetic acid. The obtained solid was filtered, washed several times with water and crystallized from methanol.

4.1.4.1. N'-(4-Oxo-1,5-diphenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)benzo-hydrazide ***7a***

The reaction time was 6h, yield 56% (2.36 g), m.p.: 179-181°C. IR $\upsilon_{max,}$ cm^{-1}: 3318 (NH), 3070 (Ar-CH), 1685 (br 2 C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.21 (d, 1H, *J* = 7.48 Hz, Ar-H), 7.35-7.52 (m, 8H, Ar-H), 7.58-7.63 (m, 2H, Ar-H), 7.74 (t, 2H, *J* = 7.60 Hz, Ar-H), 7.96 (d, 2H, *J* = 7.48 Hz, Ar-H), 8.47 (s, 1H, $H3_{pyrazole}$), 11.23 (s, 1H, NH, exchanged with D_2O), 12.96 (s, 1H, NH, exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): 105.7 ($C4_{pyrazole}$), 121.3, 122.0, 124.4, 125.3, 126.9, 127.8, 128.8, 129.9, 131.2, 132.1, 132.6, 133.2, 137.3, 137.8, 148.7 (Ar-C), 167.8 ($C=O_{pyrimidinone}$), 171.3 (C=O). MS m/z (%): 422.21 (M^+, 12.56), 404.19 (100.00). Anal.Calcd. for $C_{24}H_{18}N_6O_2$ (422.15): C, 68.24; H, 4.29; N, 19.89. Found: C, 68.37; H, 4.36; N, 20.02.

4.1.4.2. 4-Methoxy-N'-(4-oxo-1,5-diphenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)benzohydrazide ***7b***

The reaction time was 3h, yield 78% (3.52 g), m.p.: 197-200°C. IR $\upsilon_{max,}$ cm^{-1}: 3255 and 3209 (2NH), 3062 (Ar-CH), 2935 (aliph-CH), 1685 (br 2 C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 4.04 (s, 3H, OCH_3), 7.20 (t, 1H, *J* = 7.36 Hz, Ar-H), 7.34 (t, 1H, *J* = 7.26 Hz, Ar-H), 7.42 (t, 2H, *J* = 7.78 Hz, Ar-H) 7.49 (t, 2H, *J* = 7.72 Hz, Ar-H), 7.59-7.71 (m, 4H, Ar-H), 8.01 (d, 2H, *J* = 7.96 Hz, Ar-H), 8.06 (d, 2H, *J* = 7.96 Hz, Ar-H), 8.21 (s, 1H, $H3_{pyrazole}$), 9.74 (s, 1H, NH, exchanged with D_2O), 11.20 (s, 1H, NH, exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): 55.1 (OCH_3), 104.4 ($C4_{pyrazole}$), 110.7, 113.5, 118.5, 119.6, 123.2, 126.5, 127.4, 129.1, 130.2, 131.8, 143.1, 149.0, 150.7, 160.9 (Ar-C), 163.7 (C=O), 171.0 (C=O). Anal.Calcd. for $C_{25}H_{20}N_6O_3$ (452.16): C, 66.36; H, 4.46; N, 18.57. Found: C, 66.62; H, 4.50; N, 18.74.

4.1.4.3. 4-Chloro-N'-(4-oxo-1,5-diphenyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-6-yl)benzohydrazide ***7c***

The reaction time was 4h, yield 59% (2.69 g), m.p.: 215-217°C. IR $\upsilon_{max,}$ cm^{-1}: 3421 and 3387 (2 NH), 3100 (Ar-CH), 1786 and 1720 (2 C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.33 (t, 1H, *J* = 8.44 Hz, Ar-H), 7.46 (t, 1H, *J* = 7.90 Hz, Ar-H), 7.51-7.66 (m, 4H, Ar-H), 7.72 (d, 2H, *J* = 8.56 Hz, Ar-H), 7.95 (d, 2H, *J* = 8.48 Hz, Ar-H), 8.01 (d, 2H, *J* = 7.80 Hz, Ar-H), 8.16 (d, 2H, *J* = 8.60 Hz, Ar-H), 8.47 (s, 1H, $H3_{pyrazole}$), 13.17 (s, 2H, 2NH, exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): 105.4 ($C4_{pyrazole}$), 123.4,

126.4, 129.1, 130.6, 131.4, 131.7, 131.8, 132.3, 132.7, 133.5, 133.8, 134.9, 139.3, 140.5, 141.2, 152.8 (Ar-C), 154.5 ($C=O_{pyrimidinone}$), 169.2 (C=O). MS m/z (%): 456.14 (M^+, 3.63), 458.86 ($M^+ + 2$, 1.11), 40.15 (100.00). Anal.Calcd. for $C_{24}H_{17}ClN_6O_2$ (456.11): C, 63.09; H, 3.75; N, 18.39. Found: C, 63.31; H, 3.73; N, 18.52.

*4.1.5. 6-(5-Hydroxy-3-methyl-1H-pyrazol-1-yl)-1,5-diphenyl-1,5-dihydro-4H-pyrazolo [3,4-d]pyrimidin-4-one **8***

A mixture of compound **5** (3.18g, 10 mmol), ethyl acetoacetate (1.3g, 10 mmol) and anhydrous potassium carbonate (2.07g, 15 mmol) in ethanol (30 mL) was heated under reflux for 11h. The reaction mixture was cooled and the precipitated solid was collected by filteration, washed with water, dried and crystallized from ethanol to give white crystalline solid. Yield %: 53 (2.01 g), m.p.: 149-152°C. IR $\upsilon_{max,}$ cm^{-1}: 3394 (br –OH), 3094, 3051(Ar-CH), 2981, 2928 (aliph-CH), 1709 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 2.41 (s, 3H, CH_3), 7.29 (t, 1H, *J* = 7.36 Hz, Ar-H), 7.45 (t, 2H, *J* = 7.80 Hz, Ar-H), 7.63-7.74 (m, 7H, 6 Ar-H, and OH exchanged with D_2O), 7.98 (d, 2H, *J* = 7.96 Hz, Ar-H), 8.40 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 12.3 (CH_3), 103.1 ($C4_{pyrazole}$), 121.1, 126.8, 127.9, 129.5, 130.2, 132.2, 137.0, 139.1, 150.2, 151.2, 151.4 (Ar-C), 152.4 (C=O). MS m/z (%): 384.18 (M^+, 3.59), 137.08 (100.00). Anal.Calcd. for $C_{21}H_{16}N_6O_2$ (384.13): C, 65.62; H, 4.20; N, 21.86. Found: C, 65.71; H, 4.25; N, 21.99.

*4.1.6. General procedure for preparation of **9a-c**.*

A mixture of compound **5** (3.18 g, 10 mmol) and different aliphatic carboxylic acid (30 mL) was heated under reflux for the appropriate time 6-10 h. After evaporation of the excess acid under reduced pressure, the remaining solution was poured onto ice water. The obtained solid was filtered, washed with water, dried, and crystallized from ethanol.

*4.1.6.1. 1,5-Diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one **9a***

The reaction time was 10 h, yield 67% (2.19 g), m.p.: 118-119°C. IR $\upsilon_{max,}$ cm^{-1}: 3065, 3047 (Ar-CH), 1724 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.36 (t, 1H, *J* = 7.40 Hz, Ar-H), 7.52-7.58 (m, 3H, Ar-H), 7.68 (t, 2H, *J* = 7.82 Hz, Ar-H), 7.95 (d, 2H, *J* = 7.72 Hz, Ar-H), 8.10 (d, 2H, *J* = 7.80 Hz, Ar-H) 8.45 (s, 1H, $H3_{pyrazole}$), 9.36 (s, 1H, $H3_{triazole}$).

^{13}C NMR (DMSO-d_6, 100 MHz): 103.0 ($C4_{pyrazole}$), 121.5, 124.0, 127.3, 129.3, 129.7, 130.1, 133.0, 137.0, 138.7, 142.3 (Ar-C), 152.4 (C=O). MS m/z (%): 328.15 (M^+, 17.67), 77.05 (100.00). Anal.Calcd. for $C_{18}H_{12}N_6O$ (328.11): C, 65.85; H, 3.68; N, 25.60. Found: C, 65.97; H, 3.72; N, 25.89.

4.1.6.2. 8-Methyl-1,5-diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one ***9b***

The reaction time was 6 h, yield 81% (2.77 g), m.p.: 124-125°C. IR $\upsilon_{max,}$ cm^{-1}: 3055 (Ar-CH), 2982, 2928 (aliph-CH), 1712 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 2.41 (s, 3H, CH_3) 7.29 (t, 1H, *J* = 7.38 Hz, Ar-H), 7.45 (t, 2H, *J* = 7.88 Hz, Ar-H), 7.63-7.70 (m, 3H, Ar-H), 7.73 (d, 2H, *J* = 7.52 Hz, Ar-H), 7.98 (d, 2H, *J* =7.92 Hz, Ar-H), 8.40 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 12.3 (CH_3), 103.2 ($C4_{pyrazole}$), 121.1, 121.2, 122.3, 126.8, 127.9, 128.9, 129.5, 130.2, 130.4, 132.2, 136.6, 137.0, 139.1, 150.2, 151.2, 151.4 (Ar-C), 152.4 (C=O). MS m/z (%): 342.14 (M^+, 17.32), 77.07 (100.00). Anal.Calcd. for $C_{19}H_{14}N_6O$ (342.12): C, 66.66; H, 4.12; N, 24.55. Found: C, 66.85; H, 4.17; N, 24.72.

4.1.6.3. 8-Ethyl-1,5-diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one ***9c***

The reaction time was 7 h, yield 86% (3.06 g), m.p.: 133-134°C. IR $\upsilon_{max,}$ cm^{-1}: 3067, 3055 (Ar-CH), 2978, 2944, 2920 (aliph-CH), 1712 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): 1.33 (t, 3H, *J* = 7.48 Hz, CH_3), 2.78 (q, 2H, *J* = 7.48 Hz, -CH_2-), 7.21 (t, 1H, *J* = 7.38 Hz, Ar-H), 7.35 (t, 2H, *J* = 7.82 Hz, Ar-H), 7.48 (d, 2H, *J* = 7.72 Hz, Ar-H), 7.60-7.65 (m, 3H, Ar-H), 8.01 (d, 2H, *J* =7.96, Ar-H), 8.31 (s, 1H, $H3_{pyrazole}$).^{13}C NMR ($CDCl_3$, 100 MHz): 10.4 (CH_3), 19.7 (CH_2), 103.2 ($C4_{pyrazole}$), 120.8, 126.1, 127.1, 128.8, 130.1, 130.3, 131.4, 136.5, 138.9, 149.3, 151.8, 151.9 (Ar-C), 154.5 (C=O). MS m/z (%): 356.17 (M^+, 12.05), 77.05 (100.00). Anal.Calcd. for $C_{20}H_{16}N_6O$ (356.14): C, 67.40; H, 4.53; N, 23.58. Found: C, 67.62; H, 4.61; N, 23.70.

4.1.7. General procedure for preparation of ***10a-c****.*

A mixture of compound **5** (3.18 g, 10 mmol) and the appropriate benzoyl chloride (12 mmol) in dry pyridine (10 mL) was heated under reflux for 24 h. The reaction mixture was cooled and the formed precipitate was filtered, washed with water then ethanol, dried and crystallized from ethanol.

4.1.7.1. 1,5-Diphenyl-8-phenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one ***10a***

Yield 59% (2.38 g), m.p.: 218-220°C. IR $\upsilon_{max,}$ cm^{-1}: 3059, 3017 (Ar-CH), 1721 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.30 (t, 1H, *J* = 7.20 Hz, Ar-H), 7.44-7.59 (m, 12H, Ar-H), 8.01 (d, 2H, *J* = 8.40 Hz, Ar-H), 8.46 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 103.2 ($C4_{pyrazole}$), 121.1, 125.3, 126.9, 128.5, 129.3, 129.5, 130.1, 130.4, 131.9, 132.9, 137.1, 139.1, 150.7, 151.5, 151.6 (Ar-C), 152.4 (C=O). Anal.Calcd. for $C_{24}H_{16}N_6O$ (404.15): C, 71.28; H, 3.99; N, 20.78. Found: C, 71.49; H, 4.06; N, 20.96.

4.1.7.2. 8-(4-Methoxyphenyl)-1,5-diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a] pyrimidin-4(5H)-one ***10b***

Yield 79% (3.42 g), m.p.: 229-231°C. IR $\upsilon_{max,}$ cm^{-1}: 3082, 3063 (Ar-CH), 2965, 2926 (aliph-CH), 1720 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 3.79 (s, 3H, OCH_3), 7.00 (d, 2H, *J* = 8.40 Hz, Ar-H), 7.29 (t, 1H, *J* = 7.16 Hz, Ar-H), 7.42-7.46 (m, 4H, Ar-H), 7.60 (s, 5H, Ar-H) 8.00 (d, 2H, *J* = 7.92 Hz, Ar-H), 8.43 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 55.9 (OCH_3), 103.2 ($C4_{pyrazole}$), 114.8, 117.2, 121.0, 126.8, 128.5, 129.5, 130.1, 130.4, 131.0, 133.1, 137.1, 139.1, 150.7, 151.3, 151.6 (Ar-C), 162.0 (C=O). MS m/z (%): 434.22 (M^+, 3.83), 77.06 (100.00). Anal.Calcd. for $C_{25}H_{18}N_6O_2$ (434.17): C, 69.11; H, 4.18; N, 19.34. Found: C, 69.34; H, 4.25; N, 19.49.

4.1.7.3. 8-(4-Chlorophenyl)-1,5-diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one ***10c***

Yield 75% (3.28 g), m.p.: 229-231°C. IR $\upsilon_{max,}$ cm^{-1}: 3060 (Ar-CH), 1723 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.30 (t, 1H, *J* = 7.40 Hz, Ar-H), 7.46 (t, 1H, *J* = 7.80 Hz, Ar-H), 7.51 (d, 1H, *J* = 8.80 Hz, Ar-H), 7.56-7.60 (m, 3H, Ar-H), 7.71 (d, 2H, *J* = 8.40 Hz, Ar-H), 7.94 (d, 2H, *J* = 8.00 Hz, Ar-H), 8.01 (d, 2H, *J* = 8.00 Hz, Ar-H), 8.16 (d, 2H,

J = 8.40 Hz, Ar-H), 8.47 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 103.2 ($C4_{pyrazole}$), 121.1, 125.3, 126.9, 128.5, 129.3, 129.5, 129.6, 130.1, 130.4, 131.9, 132.9, 137.2, 139.1, 150.8, 151.6 (Ar-C), 152.4 (C=O). MS m/z (%): 438.14 (M^+, 11.04), 440.16 ($M^+ + 2$, 4.69), 77.06 (100.00). Anal.Calcd. for $C_{24}H_{15}ClN_6O$ (438.10): C, 65.68; H, 3.45; N, 19.15. Found: C, 65.84; H, 3.41; N, 19.34.

*4.1.8. 8-Chloromethyl-1,5-diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one **11***

A mixture of **5** (3.18 g, 10 mmol) and chloroacetyl chloride (1.38 g, 12 mmol) in acetic acid (15 mL) was heated under reflux for 4 h. The reaction mixture was cooled, poured onto water (50 mL) and the solid was filtered, dried and crystallized from acetic acid. Yield: 55.1% (2.10 g), m.p: 254-256 °C. IR $\upsilon_{max,}$ cm^{-1}: 3098, 3063, 3046, (Ar-CH), 2972 (aliph-CH), 1717 (C=O). ^1H NMR ($CDCl_3$, 400 MHz): 4.66 (s, 2H, -CH_2-), 7.25 (t, 1H, *J* = 7.22 Hz, Ar-H), 7.38 (t, 2H, *J* = 7.78 Hz, Ar-H), 7.60-7.68 (m, 5H, Ar-H), 8.01 (d, 2H, *J* = 8.08 Hz, Ar-H), 8.33 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 48.5 (CH_2), 103.3 ($C4_{pyrazole}$), 121.3, 127.0, 127.9, 129.6, 130.3, 130.8, 131.7, 137.2, 139.0, 149.7 (Ar-C), 151.4 (C=O). Anal.Calcd. for $C_{19}H_{13}ClN_6O$ (370.08): C, 60.56; H, 3.48; N, 22.30. Found: C, 60.67; H, 3.51; N, 22.48.

*4.1.9. General procedure for preparation of **12a,b.***

A mixture of **11** (3.76 g, 10 mmol), the appropriate alicyclic amine (20 mmol) and anhydrous potassium hydroxide (0.1 g) in dioxane (50 mL) was refluxed for 24 h. The reaction mixture was cooled and poured onto ice water. The formed precipitate was filtered, washed with water, dried and crystallized from ethanol to afford **12a,b.**

*4.1.9.1. 8-Morpholinomethyl-1,5-diphenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a] pyrimidin-4(5H)-one **12a***

Yield: 65% (2.77 g), m.p: 261-263 °C. IR $\upsilon_{max,}$ cm^{-1}: 3094, 3051 (Ar-CH), 2955, 2928, 2855 (aliph-CH), 1717 (C=O). ^1H NMR ($CDCl_3$, 400 MHz): 2.52-2,54 (m, 4H, $H_{2,6\ morpholine}$), 3.59-3.61 (m, 4H, $H_{3,5\ morpholine}$), 3.65 (s, 2H, -CH_2-), 7.27 (t, 2H, *J* = 6.82 Hz, Ar-H), 7.41 (t, 2H, *J* = 7.86 Hz, Ar-H), 7.62-7.69 (m, 4H, Ar-H), 8.06 (d, 2H, *J* = 7.92

Hz, Ar-H), 8.38 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 52.5 (CH_2), 52.8 (N-$CH_{2\ morpholinyl}$), 66.4 (O-$CH_{2\ morpholinyl}$), 103.2 ($C4_{pyrazole}$), 121.2, 126.8, 127.7, 127.9, 129.6, 130.0, 130.2, 130.4, 132.2, 132.7, 137.0, 139.1, 150.2, 150.5, 151.2, 151.5 (Ar-C), 152.4 (C=O). MS m/z (%): 427.17 (M^+, 1.56), 40.14 (100.00). Anal.Calcd. for $C_{23}H_{21}N_7O_2$ (427.18): C, 64.63; H, 4.95; N, 22.94. Found: C, 64.89; H, 5.04; N, 23.17.

4.1.9.2. 1,5-Diphenyl-8-(4-phenylpiperazinyl)methyl-1H-pyrazolo [4,3-e][1,2,4]triazolo [4,3-a]pyrimidin-4(5H)-one ***12b***

Yield: 73 % (3.66 g), m.p > 300 °C. IR $\upsilon_{max,}$ cm^{-1}: 3060 (Ar-CH), 2926, 2854, 2826 (aliph-CH), 1715 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): 2.69 (br s, 4H, $H_{2,6\ piperazine}$), 3.10 (br s , 4H, $H_{3,5\ piperazine}$), 3.71 (s, 2H, -CH_2-), 6.89-6.94 (m, 3H, Ar-H), 7.41-7.51 (m, 6H, Ar-H), 7.63-7.69 (m, 4H, Ar-H), 8.06-8.08 (m, 2H, Ar-H), 8.39 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR ($CDCl_3$, 100 MHz): 49.7 (piperazine-C), 52.0 (CH_2), 52.8 (piperazine-C), 103.4 ($C4_{pyrazole}$), 116.1, 116.7, 117.3, 117.2, 120.1, 120.6, 121.1, 126.3, 126.7, 126.9, 127.2, 128.9, 129.1, 129.3, 129.4, 130.3, 131.5, 136.7, 149.4, 150.1 (Ar-C), 152.0 (C=O). Anal.Calcd. for $C_{29}H_{26}N_8O$ (502.22): C, 69.31; H, 5.21; N, 22.30. Found: C, 69.46; H, 5.27; N, 22.47.

4.1.10. General procedure for preparation of ***13a-c***

A mixture of compound **5** (3.18 g, 10 mmol) and the substituted isothiocyante (15 mmol) in pyridine (10 mL) was refluxed for 12-24 h. The solution was cooled, poured onto ice water and acidified with acetic acid. The formed precipitate was filtered, washed several times with water and crystallized from ethanol to afford the desired compounds.

4.1.10.1. 8-Methylamino-1,5-diphenyl-1H-pyrazolo[4,3e][1,2,4]triazolo[4,3-a]pyrimidin -4(5H)-one ***13a***

The reaction time was 12 h, yield: 60 % (2.14 g), m.p: 184-186 °C. IR $\upsilon_{max,}$ cm^{-1}: 3321 (NH), 3066, 3034 (Ar-CH), 2924, 2853 (aliph-CH), 1701 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 2.88 (s, 3H, -CH_3), 6.26 (s, 1H, NH, exchanged with D_2O), 7.08 (t, 1H, J = 7.20 Hz, Ar-H), 7.23 (t, 2H, J = 7.20 Hz, Ar-H), 7.45-7.54 (m, 5H, Ar-H), 7.88 (d, 2H, J = 7.60 Hz, Ar-H), 8.09 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 34.0 (CH_3),

108.4 ($C4_{pyrazole}$), 125.5, 125.6, 130.8, 132.2, 133.6, 134.7, 134.9, 135.5, 140.8, 140.9, 143.9, 153.3, 156.1, 156.7, 157.2 (C=O). MS m/z (%): 357.14 (M^+, 100.00), 358.14 (M^++1, 25.19). Anal.Calcd. for $C_{19}H_{15}N_7O$ (357.14): C, 63.26; H, 4.23; N, 27.44. Found: C, 63.02; H, 4.32; N, 27.65.

4.1.10.2. 8-Ethylamino-1,5-diphenyl-1H-pyrazolo[4,3e][1,2,4]triazolo[4,3-a]pyrimidin-4(5H)-one ***13b***

The reaction time was 12 h, yield: 55 % (2.04 g), m.p: 193-195 °C. IR $\upsilon_{max,}$ cm^{-1}: 3219 (NH), 3080 (Ar-CH), 2971 (aliph-CH), 1697 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 1.20 (t, 3H, *J* = 7.00 Hz, CH_3), 3.30 (q, 2H, *J* = 6.01 Hz, CH_2), 6.85 (t, 1H, *J* = 5.20 Hz, NH exchanged with D2O), 7.26 (t, 1H, *J* = 7.20 Hz, Ar-H), 7.42 (t, 2H, *J* = 7.40 Hz, Ar-H), 7.62-7.68 (m, 5H, Ar-H), 7.98 (d, 2H, *J* = 8.00 Hz, Ar-H), 8.32 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 14.7 (CH_3), 37.5 (CH_2), 103.6 ($C4_{pyrazole}$), 120.9, 126.6, 128.3, 129.5, 130.3, 131.1, 136.6, 139.2, 149.3, 151.5, 151.6 (Ar-C), 152.0 (C=O). MS m/z (%): 371.14 (M^+, 74.95), 185.96 (100.00). Anal.Calcd. for $C_{20}H_{17}N_7O$ (371.15): C, 64.68; H, 4.61; N, 26.40. Found: C, 64.79; H, 4.68; N, 26.58.

4.1.10.3. 1,5-Diphenyl-8-phenylylamino-1H-pyrazolo[4,3e][1,2,4]triazolo[4,3-a] pyrimidin-4(5H)-one ***13c***

The reaction time was 24 h, yield: 63 % (2.64 g), m.p: 241-242 °C. IR $\upsilon_{max,}$ cm^{-1}: 3321 (NH), 3066, 3034 (Ar-CH), 2924, 2853 (aliph-CH), 1701 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.07 (t, 1H, *J* = 7.32 Hz, Ar-H), 7.28 (t, 1H, *J* = 7.42 Hz, Ar-H), 7.37 (t, 2H, *J* = 7.84 Hz, Ar-H), 7.44 (t, 2H, *J* = 7.86 Hz, Ar-H), 7.65-7.77 (m, 7H, Ar-H), 8.00 (d, 2H, *J* = 8.04 Hz, Ar-H), 8.37 (s, 1H, $H3_{pyrazole}$), 9.09 (s, 1H, NH, exchanged with D_2O). ^{13}C NMR (DMSO-d_6, 100 MHz): 103.6 ($C4_{pyrazole}$), 119.8, 120.9, 123.2, 126.7, 128.7, 129.2, 129.5, 130.2, 130.5, 131.2, 136.8, 139.2, 139.4, 149.0, 149.1, 151.4 (Ar-C), 151.8 (C=O). MS m/z (%): 419.14 (M^+, 32.33), 77.05 (100.00). Anal. Calcd. for $C_{24}H_{17}N_7O$ (419.15): C 68.72; H, 4.09; N, 23.38. Found: C, 69.01; H, 4.15; N, 23.52.

4.1.11. 1,5-Diphenyl-8-thioxo-7,8-dihydro-1H-pyrazolo[4,3-e[]1,2,4]pyrimidin-4(5H)-one ***14***

To a mixture of **5** (3.18 g, 10 mmol) and potassium hydroxide (0.2g) in absolute ethanol (20 mL) at 0° C, was added carbon disulfide (1.53 g, 20 mmol) in one portion. The resulting mixture was refluxed with stirring for 6 hr. The solvent was evaporated under reduced pressure and the residue was acidified with 2M hydrochloric acid. The formed solid was filtered, washed with water and crystallized from ethanol. Yield: 62.4% (2.24 g), m.p**:** 178-81°C. IR $\upsilon_{max,}$ cm^{-1}: 3395 (NH), 3063 (Ar-CH), 1693 (C=O), 1207 (C=S).^{1}H NMR (DMSO-d_6, 400 MHz): 7.24 (t, 1H, *J* = 7.32 Hz, Ar-H), 7.39-7.47 (m, 4H, 3 Ar-H and NH exchanged with D_2O), 7.54 (t, 2H, *J* = 7.60 Hz, Ar-H), 7.62 (d, 2H, *J* = 7.76 Hz, Ar-H), 8.04 (d, 2H, *J* = 8.04 Hz, Ar-H), 8.23 (s, 1H, $H3_{pyrazole}$). ^{13}C NMR (DMSO-d_6, 100 MHz): 103.1 ($C4_{pyrazole}$), 120.6, 126.2, 128.3, 128.7, 128.8, 129.4, 135.0, 136.6, 139.5, 150.1, 150.8 (Ar-C), 152.1 (C=O), 167.8 (C=S). MS m/z (%): 360.19 (M^+, 0.58), 44.03 (100.00). Anal.Calcd. for $C_{18}H_{12}N_6OS$ (360.08): C 59.99; H, 3.36; N, 23.32. Found: C, 60.17; H, 3.34; N, 23.57.

4.2. In-vitro enzyme inhibitory assay

The mechanism of kinase inhibition was investigated using a cell-free assay with commercially available recombinant Src, PI3K, Abl/mutant T315I according to the reported procedure [21]. Activity was measured using their respective specific peptide substrates. Reaction conditions were: 0.012 µM [γ^{-32}P]ATP, peptide 50 µM (Abl), 250 µM (Src), or 200 µM (PI3K) and 0.08 µM Src, 0.15 µMPI3K or 0.022 µM c-Abl/T315I Abl. Dose-response curves were generated by fitting the data by computer simulation to the following equation: $E_{(\%)} = E_{max}/ (1+ [I]/ID_{50})$, where $E_{(\%)}$ is the fraction of the enzyme activity measured in the presence of the inhibitor, E_{max} is the activity in the absence of the inhibitor, [I] is the inhibitor concentration and ID_{50} is the inhibitor concentration at which $E_{(\%)} = 0.5\ E_{max}$. Each experiment was done in two independent replicates and mean values were used for the interpolation.

4.3. In-vitro cytotoxicty MTT assay

Antiproliferative activity of the target compounds was determined in cells treated with the different concentrations of the tested compounds in comparison with untreated control using MTT assay as follows**:**

The cells were grown on a medium supplemented with 10% inactivated fetal bovine serum. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. Different concentrations of 100, 10, 1.0, 0.1 and 0.01 micro mole (μM) of each compound were tested for cytotoxicity. Tetraplicate wells were prepared for each concentration in addition to cell control (cell only without compounds). Culture media containing different concentration of tested compounds and dead cells were decanted leaving only viable attached cells into the tissue culture plate. MTT reagent (40 μl) was added to each well including blank and negative control wells. The plates were incubated in dark for 4 hrs for the reduction of MTT into formazan (purple needle color) by dehydrogenase activity in mitochondria of viable cells. The absorbance was measured at 570 nm, then the percentage of cell survival was calculated. The inhibitory concentration 50 (IC_{50}) was calculated by plotting log molar concentration of the tested compounds against survival rate percent.

4.4. Molecular Docking

X-ray crystal structure of the three dimensional X-ray crystal structure of axitinib in complex with the wild type human bcr-Abl kinase (PDB code: 4WA9), its T315I gatekeeper mutant (PDB code: 4TWP) and c-Src (PDB code: 4O2P) were downloaded from http://www.rscb.org/pdb. All molecular modeling calculations and docking studies were carried out using Discovery Studio software v4.0.0.13259 running on a Windows7 PC [37].

4.4.1. Binding Site Sphere Determination

The protein-ligand complexes obtained from the protein data bank were prepared for docking as follows: deletion of one of the protein chains, deletion of all the co-crystallized water molecules. Automatic protein preparation module was used applying

CHARMm forcefield. The binding site sphere has been defined automatically by the software.

4.4.2. Preparation of Target Compounds for Docking

The docked compounds were prepared for docking by applying the following protocol; 2D structures of the docked ligands were built using Marvin Sketch and copied to Discovery Studio 4. Ligands were prepared using "Prepare Ligands" protocol in Discovery Studio where hydrogen atoms were added at their standard geometry, optical isomers and 3D conformations were automatically generated.

4.4.3. Running Docking

Docking was performed using CDOCKER protocol in Discovery Studio keeping the parameters at default. The best scoring pose of the docked compounds was recognized. Receptor-ligand interactions of the complexes were examined in 2D and 3D styles.

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Highlights:

- Some pyrazolo[3,4-*d*]pyrimidines were prepared as dual Src-Abl inhibitors.
- Compounds **7a**, **7b** revealed good activity against the T315I mutant Abl and Src.
- Compound **10b** exerted equal inhibition against both Abl forms.
- **10b**, the rigide analog of **7b** showed a decreased activity against both enzymes.
-These compounds showed promising cytotoxic activity against K-562 cell line.